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Phosphatidylinositol-3,4-Bisphosphate and Its Binding Protein Lamellipodin Regulate Chemotaxis of Malignant B Lymphocytes

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Cell migration is controlled by PI3Ks, which generate lipid messengers phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2] and consequently recruit pleckstrin homology (PH) domain–containing signaling proteins. PI3K inhibition impairs migration of normal and transformed B cells, an effect thought to partly underlie the therapeutic efficacy of PI3K inhibitors in treatment of B cell malignancies such as chronic lymphocytic leukemia. Although a number of studies have implicated phosphatidylinositol-3,4,5-trisphosphate in cell migration, it remains unknown whether PI(3,4)P2 plays a distinct role. Using the PI(3,4)P2-specific phosphatase inositol polyphosphate 4-phosphatase, we investigate the impact of depleting PI(3,4)P2 on migration behavior of malignant B cells. We find that cells expressing wild-type, but not phosphatase dead, inositol polyphosphate 4-phosphatase show impaired SDF-induced PI(3,4)P2 responses and reduced migration in Transwell chamber assays. Moreover, PI(3,4)P2 depletion in primary chronic lymphocytic leukemia cells significantly impaired their migration capacity. PI(3,4)P2 depletion reduced both overall motility and migration directionality in the presence of a stable chemokine gradient. Within chemotaxing B cells, the PI(3,4)P2-binding cytoskeletal regulator lamellipodin (Lpd) was found to colocalize with PI(3,4)P2 on the plasma membrane via its PH domain. Overexpression and knockdown studies indicated that Lpd levels significantly impact migration capacity. Moreover, the ability of Lpd to promote directional migration of B cells in an SDF-1 gradient was dependent on its PI(3,4)P2-binding PH domain. These results demonstrate that PI(3,4)P2 plays a significant role in cell migration via binding to specific cytoskeletal regulators such as Lpd, and they suggest that impairment of PI(3,4)P2-dependent processes may contribute to the therapeutic efficacy of PI3K inhibitors in B cell malignancies. The Journal of Immunology, 2016, 196: 000–000.
itors include blockade of BCR signaling and chemokine receptor signaling (5).

Class I PI3Ks catalyze the production of two 3-phosphoinositide (PI) messengers, PIP3 and phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2], both of which can transduce signals by recruiting specific pleckstrin homology (PH) domain–containing proteins to the cell membrane (22, 23). The current paradigm generally attributes the function of PI3K in cell migration to PIP3; however, in most studies the relative contributions of PIP3 and PI(3,4)P2 have not been distinguished. Although some PIP3-specific binding proteins are known to regulate cell migration (16), it remains a major unanswered question in the field whether PI(3,4)P2 is also implicated in this cellular function.

Two highly related PI(3,4)P2-specific lipid phosphatases have been identified, type I and II inositol polyphosphate-4-phosphatases (INPP4A and B). They selectively hydrolyze PI(3,4)P2 without affecting the cellular levels of other PI lipids such as PI(3,4,5)P3 or PI(4,5)P2, and PIP3 (24–27). Loss of INPP4B led to enhanced wound healing responses (a measure of adherent cell migration) in breast cancer (27) and melanoma cells (28), suggesting a correlation between increased levels of PI(3,4)P2 and increased migration capacity in these cells. In MDA-MB-231 breast cancer cells, PI(3,4)P2 was found to be enriched at the leading edge (29). However, experiments distinguishing the specific role of PI(3,4)P2 by loss-of-function approaches are lacking, and its role in directional migration within chemokine gradients had not been assessed.

The PH domain–containing protein lamellipodin (Lpd), also known as Ras association and PH domains 1, is proposed to be a specific PI(3,4)P2-binding protein (29–32); however, one dissenting study proposed that its PH domain might not be effective in PI binding (33). Lpd was shown to mediate random motility in breast cancer cells (29, 34) as well as extension of neuronal processes (35). It remains unknown whether Lpd is involved in directional migration in response to specific chemotactic stimuli, and whether the function of Lpd in promoting cell migration depends on its PH-domain–binding PH domain.

In this work, we provide evidence that depletion of PI(3,4)P2 impairs the motility and directionality of B cell chemotaxis. We further find that Lpd colocalizes with PI(3,4)P2 in chemotaxing B cells and mediates migration directionality in a PH domain–dependent manner. To our knowledge, these results provide the first demonstration that PI(3,4)P2 and its binding protein Lpd are elements of the PI3K-dependent cell guidance mechanism.

Materials and Methods

Plasmid constructs

Constructs for INPP4 or Lpd overexpression or Lpd knockdown (KD) were generated as described below. The expression cassette of enhanced GFP (EGFP)–tagged human INPP4A, between Ase I and Mlu I sites of the pEGFP-C2-4-phosphatase plasmid (provided by C. Mitchell) (25), was (EGFP)–tagged human INPP4A, between Ase I and Mlu I sites of the pEGFP-C2-4-phosphatase plasmid (provided by C. Mitchell) (25), was transferred into pCDH-CMV-MCS-EF1-Puro (System Biosciences). An untagged INPP4A vector was generated as described below. The expression cassette of enhanced GFP

FIGURE 1. WT, but not phosphatase-dead, INPP4A inhibits malignant B cell migration. INPP4A WT or catalytically inactive mutant (Inactive) was overexpressed in RAJI cells through lentiviral transduction. (A) Similar expression levels of WT and inactive INPP4A was confirmed by Western blot. (B) INPP4A expression does not significantly affect cell viability. RAJI cell expressing inactive or WT INPP4A were stained for annexin V and with DAPI and analyzed by flow cytometry. (C and D) Active INPP4A impairs migration of RAJI cells in Transwell chamber assays. RAJI cells transfected with empty vector (control) or expressing inactive or WT INPP4A were assayed for Transwell migration toward 100 ng/ml SDF-1/CXCL12 (C) or 500 ng/ml BLC/CXCL13 (D). Results are normalized to control cells and represent mean ± SD of three independent experiments. *p < 0.05.

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Western blotting
Abs used for Western blotting were goat anti-INPP4A (Abcam, EPR3426), rabbit anti-INPP4B (Abcam, EPR3108Y), rabbit anti-Lpd (Santa Cruz Biotechnology, sc-68380) and anti-GAPDH (Trevigen, 2275-PC-100). Western blots were performed as described (38).

Flow cytometry
PI(3,4)P2 staining was performed according to a recently published method (26). Briefly, cells were stimulated as indicated, fixed with PBS containing 1% BSA and 2% PFA (30 min at room temperature), washed, permeabilized with PBS containing 1% BSA and 0.5% saponin (30 min at room temperature), washed, and stained overnight with 2 μg/ml PI(3,4)P2-specific mAb (Echelon Biosciences, Z-P034b). Staining was detected using Alexa Fluor 488–or 647–labeled secondary Abs (Life Technologies, A11029 or A21236) and mean fluorescence intensity was measured by flow cytometry analysis on a FACSCanto II instrument (BD Biosciences). PI(3,4)P2 was similarly stained using a specific mAb (Echelon Biosciences, Z-P345b).

Mass spectrometry
Cells (7 × 10^6) in 0.34 ml culture medium were stimulated with SDF-1 for the indicated times. Incubations were terminated with 1.5 ml methanol/ chloroform/1 M HCl (20:9:1 [v/v/v]). The sample was then stored in dry ice for 3–7 d before analysis. After warming to room temperature, samples were split into two aliquots, one for analysis of PI(3,4)P2 and one for analysis of PI(3,4)P2. Ten nanograms C16:0-C17:0 PI(3,4)P2 ISD, 100 ng C16:0-C17:0 PI(3,4,5)P3 ISD (for the PI(3,4,5)P3 measurements), or 50 ng d5PI(3,4)P2 ISD, 250 ng d6PI(4,5)P2 ISD, and 50 ng C17:0-C20:4 PI ISD [for the PI(3,4)P2 measurements] were spiked in as internal standards (in volumes ≤ 10 μl) to correct for lipid recovery. Then, to each ~0.75 ml aliquot, 725 μl chloroform and 170 μl 2 M HCl were added to create a Folch phase partition; the lower phase was recovered and methylated using trimethylsilyldiazomethane, as described in Clark et al. (39). The methylated phosphoinositides were then separated and analyzed by HPLC–electrospray ionization mass spectrometry using a C4 column for the measurement of C38:4 species of PI, PIP, PIP2, and PIP3, as described by Kielkowska et al. (40), and a C18 column for the separation of C38:4 species of PI(3,4)P2 and PI(4,5)P2 (A. Kielkowska, unpublished observations). The data for each phosphoinositide species was corrected for the recovery of its internal standard.

Transwell migration assay
These assays were performed essentially as described (38), with the following modifications. For RAJI, 1–2 × 10^5 cells were loaded into Transwell chambers with 8 μm pore size, and 9–10 h migration time was used. For CLL, 1 × 10^6 cells were loaded into chambers with 5 μm pore size, and 5–6 h migration time was used. Flow cytometry was used to count transfected (GFP+) cells present in the top and bottom chambers.

Microfluidic device chemotaxis assay and confocal microscopy analyses
Analyses of cell migration within microfluidic devices generating a stable SDF-1 gradient were performed essentially as described (38, 41). Briefly, RAJI cells were adhered to Y-shaped microfluidic devices coated with fibronectin (50 μg/ml; BD Biosciences) and allowed to migrate under a 100 nM SDF-1 gradient. Cell movement was recorded by time-lapse imaging using a CSU-X1M 5000 spinning disc confocal microscope (Carl Zeiss Canada). Cell tracking analyses were performed on a FACSCanto II instrument (BD Biosciences). PI(3,4)P2 was similarly stained using a specific mAb (Echelon Biosciences, Z-P345b).

Statistical analysis
Significance was assessed using Student t test. A p value <0.05 was considered significant.

Results
PI(3,4)P2 depletion by INPP4 inhibits B cell chemotaxis
Unlike the well-studied PI phosphatase PTEN, INPP4 hydrolyzes PI(3,4)P2 but not PIP3 (24, 25). We thus reasoned that INPP4 can provide a genetic tool to deplete PI(3,4)P2 and probe the functions of this lipid messenger in cell migration. INPP4A and its catalytically inactive mutant were overexpressed in RAJI cells, a human B lymphoma cell line (Fig. 1A). Transfectants did not show reduced cell viability (Fig. 1B). Transwell chamber migration

**FIGURE 2.** INPP4A significantly reduces cellular PI(3,4)P2 levels. RAJI transfectants were serum starved and stimulated with 100 ng/ml SDF-1 for the indicated times and PI(3,4)P2 or PI(3,4,5)P3 levels were determined by flow cytometry or mass spectrometry methods. (A and C) PI(3,4)P2 levels as assessed by staining with anti-PI(3,4)P2 or anti-PI(3,4,5)P3 mAb, and subsequent flow cytometry analysis. Results are expressed as percentage change in mean fluorescence intensity (MFI) relative to control (unstimulated cells expressing inactive INPP4A). Graphs represent mean ± SEM from four experiments. (B and D) PI(3,4)P2 or PI(3,4,5)P3 levels as assessed by mass spectrometry, expressed relative to PI. Results represent the average and SE of triplicate determinations and are representative of two similar experiments. PI(3,4,5)P3, PIP3. *p < 0.05, **p < 0.01.
assays demonstrated that WT INPP4A, but not inactive INPP4A, significantly inhibited migration toward SDF-1/CXCL12 (Fig. 1C) or BLC/CXCL13 (Fig. 1D). We assessed the impact of INPP4A activity on levels of PI(3,4)P2 and PI(3)P generated after SDF-1 stimulation using both flow cytometry (26, 29) and mass spectrometry methods (Fig. 2, Supplemental Fig. 1). Whereas INPP4A severely blunted the SDF-induced increases in PI(3,4)P2 (Fig. 2A, 2B), PI(3)P responses appear less affected (Fig. 2C, 2D). Taken together, these results indicate that INPP4A-mediated depletion of PI(3,4)P2 can functionally impact B cell migration.

Depletion of PI(3,4)P2 in primary CLL cells impairs their migration capacity

Inhibition of PI3K activity has been shown to effectively block migration of CLL cells, an effect that may partly underlie the efficacy of newly approved therapeutics targeting the PI3K pathway in CLL (20, 21). INPP4A and INPP4B were found to be expressed at variable levels in human B cell lines and primary CLL (Supplemental Fig. 2). To determine whether PI(3,4)P2 depletion by INPP4 enzymes impacts the migration capacity of primary CLL, INPP4A or INPP4B was transiently expressed in leukemic cells isolated from CLL patients (Fig. 3A). Both INPP4A and INPP4B (26, 27) were found to significantly deplete PI(3,4)P2 in CLL cells (Fig. 3A, 3B). In every patient examined, transfectants transiently expressing WT INPP4 showed reduced migration capacity relative to the same patient cells expressing phosphatase-dead enzyme (Fig. 3C). INPP4A and INPP4B were found to have similar ability to inhibit CLL migration in a phosphatase-dependent manner (Fig. 3C). These results suggest that impairment of PI(3,4)P2-dependent processes may contribute to the therapeutic efficacy of PI3K inhibitors in B cell malignancies.

PI(3,4)P2 is required for both overall motility and directional migration within a chemokine gradient

The role of PI3K in priming of overall motility versus sensing of chemotactic gradients differs among cell types (18, 43). To characterize the role of PI(3,4)P2 in controlling the migratory behaviors of individual cells within a chemokine gradient, real-time microscopic tracking was performed in a microfluidic chemotaxis system (38, 41, 44). Control cells expressing phosphatase-dead INPP4A or PI(3,4)P2-depleted cells expressing active INPP4A were allowed to migrate on a fibronectin-coated substrate in the presence of a stable SDF-1 gradient (Fig. 4A, Supplemental Videos 1, 2). In this system, control cells display directional migration up the SDF-1 gradient and a polarized morphology with a round cell body followed by an extended uropod. In contrast, PI(3,4)P2-depleted cells show less active migration overall and frequently extend protrusions in random directions. The cell migration paths over time were tracked (Fig. 4B), and quantitative analysis of the cell tracks determined that PI(3,4)P2 depletion led to reduced migration speed and chemotactic index, a measure of migration directionality (Fig. 4C). These results indicate that PI(3,4)P2 controls both motility and directionality.

Lpd is expressed in malignant B cells and colocalizes with PI(3,4)P2 in migrating B cells in a PH domain–dependent fashion

Lpd is an actin-remodeling protein reported to bind PI(3,4)P2 (30) and to regulate random motility in nonhematopoietic cells (34). To determine whether Lpd may be a mediator of PI(3,4)P2-dependent chemotaxis in malignant B cells, we first examined expression of Lpd in human B cells and found that Lpd is expressed in a variety of malignant B cell lines and primary CLL B cells (Fig. 5A). To visualize the respective subcellular localization of PI(3,4)P2 and Lpd, EGFP-tagged WT Lpd protein (Lpd WT-GFP) or PH domain–deleted mutant (Lpd ΔPH-GFP) were overexpressed in RAJI cells, subjected to an SDF-1 gradient, and then fixed, stained with protein from RAJI cells expressing INPP4A or INPP4B, respectively. After 18–24 h, cells were serum starved, stimulated with 100 ng/ml SDF-1, and PI(3,4)P2 levels were determined by flow cytometry. Data represent mean ± SEM from three patients. (C) PI(3,4)P2 depletion inhibits the migration of primary CLL cells. Migration capacity of CLL cells expressing WT versus phosphatase-dead INPP4 proteins was compared. Eighteen to twenty-four hours after nucleofection, cells were assayed for Transwell migration of GFP-expressing cells toward 100 ng/ml SDF-1. In every case, WT INPP4-expressing cells showed reduced migration relative to the same patient cells expressing inactive mutant. Data from individual CLL patients are presented as percentage inhibition of WT relative to mutant enzyme. *p < 0.05, **p < 0.01.
FIGURE 4. Migration behavior of PI(3,4)P2-depleted cells in microfluidic chambers generating a stable SDF-1 gradient. (A) INPP4A inactive or WT RAJI cells were allowed to migrate in a microfluidic chemotaxis device under a 100 nM SDF-1 gradient (direction of gradient represented with a triangle). Cell movements were recorded using time-lapse imaging and tracked using ImageJ software. The figure shows representative cells at the indicated time points during time lapse imaging. Scale bars, 10 μm. See Supplemental Videos 1 and 2 for time-lapse imaging movies. (B) Migration tracks were normalized to a common origin (0, 0) in two-dimensional plots. Solid circles label the end of migration tracks. (C) Migration speed and chemotactic index were calculated as described in Materials and Methods. Data represent the mean ± SEM of 204 INPP4A inactive cells and 318 INPP4A WT cells from two independent experiments. ***p < 0.001, ****p < 0.0001.

not Lpd ΔPH-GFP, was apparent throughout the cell depth as determined by analysis at individual Z-planes (Supplemental Fig. 3). Membrane recruitment of WT Lpd-GFP was inhibited by coexpression of active INPP4B, but not phosphatase-dead INPP4B (Fig. 5C, 5D). Taken together, these results indicate that Lpd is recruited to the plasma membrane of migrating B cells in a PH domain– and PI(3,4)P2-dependent manner.

Time-lapse imaging of Lpd WT-GFP suggested that it preferentially accumulates toward the migrating front during chemotaxis, whereas Lpd ΔPH-GFP does not (Fig. 6A). Quantitative analysis confirmed a PH domain–dependent accumulation of Lpd at the migrating front (Fig. 6B). Analysis of three-dimensional image reconstructions further revealed a subtle asymmetric distribution of PI(3,4)P2 and Lpd WT-GFP in migrating B cells. Side views close to the cellular median plane suggested that both PI(3,4)P2 and Lpd WT-GFP were slightly enriched toward the cellular front facing the direction of cell movement, whereas this pattern was not observed with Lpd ΔPH-GFP (Fig. 6C). Taken together, these results provide the first evidence, to our knowledge, implicating Lpd as a potential downstream mediator of PI(3,4)P2 in malignant B cell migration.

Lpd mediates directional migration of malignant B cells in a PH domain–dependent manner

The function of Lpd in cell migration was assessed by overexpression of Lpd-GFP or shRNA KD of endogenous Lpd. Lpd-GFP overexpression led to increased migration toward SDF-1 in Transwell assays, whereas migration was significantly inhibited by Lpd KD using shRNAs targeting either Lpd coding sequence or 3′ UTR (Fig. 7A). To determine the role of the Lpd PH domain in controlling motility and directionality, KD rescue experiments were performed by adding back Lpd WT-GFP or Lpd ΔPH-GFP to KD-UTR cells and tracking migration behavior within a stable SDF-1 gradient (Fig. 7B–D). Under these conditions, Lpd KD cells were severely impaired in both motility and directionality (Fig. 7B–D). Expression of Lpd WT-GFP but not Lpd ΔPH-GFP in KD-UTR cells restored a similar pattern of cell tracks compared with control cells (Fig. 7B). Quantitative analysis indicated that Lpd ΔPH-GFP rescued migration speed to a similar extent as did Lpd WT-GFP (Fig. 7C), suggesting that Lpd has a function in overall cell motility that does not critically depend on its PH domain. Interestingly, however, Lpd ΔPH-GFP failed to restore migration directionality, as assessed by chemotactic index (Fig. 7D). These results indicate that Lpd controls the directionality of malignant B cell chemotaxis through its PI(3,4)P2-binding PH domain.

Discussion

Although the PI3K signaling pathway is established to play important roles in lymphocyte functions, our knowledge is limited regarding the biological functions attributable to the specific PI3K products PI(3,4)P2 versus PI(3). Loss-of-function mutations in INPP4B have been associated with some metastatic solid tumors, suggesting that specific uncontrolled accumulation of PI(3,4)P2 may contribute to development of invasive phenotypes in cancer (27, 45). Although these studies have increased interest in understanding the distinct functions of PI(3,4)P2, defining the normal biological functions of this PI requires loss-of-function approaches that do not affect PI3 as we have employed in the present study. Our results provide the first evidence to our knowledge that PI(3,4)P2 plays a unique role in chemotaxis of malignant B cells, impacting on both migration motility and directionality.

PI3K products are generally thought to be enriched at the leading edge of a migrating cell. However, most previous work used the PH domain of Akt to probe PI3K activity, which does not distinguish between PI3P and PI(3,4)P2 (46, 47). A few studies applied GFP fusions or fluorescence resonance energy transfer biosensors based on the PI(3,4)P2-specific PH domain of TAPP1 to investigate the localization of PI(3,4)P2 in migrating cells. In migrating zebrafish primordial germ cells, GFP-TAPP1 PH did not show any preferential local enrichment (48). However, during
random migration of zebrafish neutrophils, GFP-TAPP1 PH was enriched at the cell front, and (seemingly to a lesser extent) at the tail (13). In motile Madin–Darby canine kidney cells, fluorescence resonance energy transfer experiments indicated that PI(3,4)P2 signal was the most obviously accumulated at the leading edge, whereas enrichment was also seen on the plasma membrane in general (49). Our results directly examining PI(3,4)P2 distribution using a specific Ab (26, 29) suggest that whereas PI(3,4)P2 is extensively present on the plasma membrane, there appeared to be a subtle local enrichment at the front of migrating cells. The PI(3,4)P2 distribution pattern was strongly correlated with that of its binding protein Lpd. This distribution pattern may contribute to the function of PI(3,4)P2-Lpd signaling in maintaining persistent migration directionality.

Several PI(3,4)P2-binding proteins may participate in lymphocyte chemotaxis. The tandem PH domain containing proteins (TAPP1 and TAPP2) are well-documented PI(3,4)P2-binding proteins (50–52). TAPP2 was found to mediate chemotaxis of malignant B cells (38); however, its cellular localization pattern in migrating cells showed enrichment toward the cell rear and sides, suggesting that TAPP2 function in chemotaxis may involve interaction with distinct protein complexes containing utrophin and stable F-actin required for front–back cell polarity (38). In contrast, we found in the present study that Lpd colocalizes with the PI(3,4)P2 pool at the front of migrating B cells. Lpd was recently found to interact with actin remodeling protein complex WAVE and mediate migration of mesenchymal and epithelial cells in *Xenopus* and *Drosophila* models, respectively (34). To our knowledge, the present results provide the first evidence for expression of Lpd in human lymphocytes and function in directional migration driven by a chemotactic gradient. Our results further indicate that the Lpd PH domain is critical for its membrane recruitment and function in lymphocytes. Interestingly, PH domain–deleted Lpd appeared to be sufficient to restore migration speed in Lpd KD

**FIGURE 5.** Lpd is expressed in malignant B cells and colocalizes with PI(3,4)P2 in migrating B cells in a PH domain–dependent fashion. (A) Western blot showing Lpd expression in the indicated human B cell lines and in primary CLL B cells isolated from multiple patients. (B) RAJ cells expressing WT Lpd-EGFP fusion protein (Lpd WT-GFP) or the PH domain deleted version (Lpd ΔPH-GFP) were stimulated with SDF-1, fixed, intracellularly stained for PI(3,4)P2, and visualized by confocal microscopy. Representative images demonstrate coenrichment of Lpd WT-GFP, but not Lpd ΔPH-GFP, with PI(3,4)P2 on the cell membrane. Right panel, Lpd WT-GFP is significantly more colocalized with PI(3,4)P2 than is Lpd ΔPH-GFP. Pearson correlation coefficients for Lpd and PI(3,4)P2 were quantified in 10 Lpd WT-GFP and 10 ΔPH cells. (C) Lpd membrane localization is inhibited by INPP4. RAJ cells were transiently transfected with Lpd-GFP and INPP4B-mCherry (WT or inactive), and after 18 h were stimulated with SDF-1 for 5 min, fixed, and imaged. (D) Quantitative analysis of Lpd-GFP membrane localization in cells coexpressing INPP4B. Graphs show the correlation between INPP4B-mCherry expression level (measured as mCherry mean fluorescence intensity) and Lpd-GFP membrane/cytoplasmic intensity ratio in individual cells. Note significant negative correlation between WT INPP4B-mCherry expression levels and Lpd-GFP membrane localization. Results are representative of two experiments (>60 cells in total). Original magnification ×63. ***p < 0.001.
cells, suggesting PI(3,4)P2-independent function of this molecule in motility in addition to a PI(3,4)P2-dependent function in chemokine gradient sensing.

The finding that PI(3,4)P2 depletion by INPP4 impairs overall motility suggests that chemokine-induced PI(3,4)P2 generation may have a role in priming cell motility. Although Lpd is found to be required for cell motility, this did not absolutely depend on its PH domain, suggesting that other PI(3,4)P2 binding proteins may serve this function. Besides TAPP proteins discussed above, a few other PI(3,4)P2-binding proteins were reported to be involved in cell migration; however, many of these also bind to PI(3,4)P2. These include protein kinase Akt (53), switch-associated protein-70 (54), 3-phosphoinositide-dependent protein kinase-1 (55), and ARNO/cytohesin2 (56). Defining the relative contributions of PI(3,4)P2 versus PIP3 to the migration-regulatory functions of these molecules will be an important future research direction.

The inositol phosphatase SHIP produces PI(3,4)P2 via dephosphorylation of PIP3, and one study found that this enzyme is responsible for a significant proportion of the PI(3,4)P2 produced in murine B cells after BCR cross-linking (57). Several studies have implicated SHIP in cell migration. Expression of constitutively active SHIP in T cells was found to suppress chemotaxis (58), whereas SHIP-deficient mouse B cells were found to have enhanced migration (59). However, SHIP silencing in human T cells had a significant impact on the cytoskeleton and reduced rather than increased overall motility (60). The authors of this study concluded that SHIP is an important cytoskeletal regulator that functions partly via phosphatase-independent actions mediated by interacting partners such as Shc and p62dok. Consistent with that study, we found that SHIP1 KD in RAJI cells led to a ∼30% decrease in migration (data not shown). Although it would be predicted that SHIP KD would reduce PI(3,4)P2 levels, potentially contributing to this decrease, this experiment cannot distinguish effects due to PI alterations from phosphatase-independent roles of SHIP. To our knowledge, a comparison of phosphatase-dependent and independent functions of SHIP (as we have done in the present study for INPP4) has not been performed. As the product of SHIP, PI(3,4)P2 is often viewed as being downstream of PI3K-dependent phosphorylation of PI(4)P. Using our mass spectrometry method, we noted an apparent alteration in kinetics of PIP3 accumulation in INPP4A-expressing cells, suggesting that PI(3,4)P2 may potentially impinge on PIP3 generation or turnover. Understanding the dynamic interplay of PIP3 and PI(3,4)P2-dependent signaling is clearly an important future goal.

A significant body of work has established that PIP3-selective binding protein Btk plays critical roles in B cell activation, in-

FIGURE 6.  Distribution of Lpd and PI(3,4)P2 in chemotaxing B cells. (A) Lpd WT-GFP– or Lpd ΔPH-GFP–expressing RAJI cells migrating in the presence of a SDF-1 gradient were observed by time-lapse live cell imaging. Original magnification ×10 differential interference contrast and confocal microscopy. White arrowheads in the differential interference contrast image series identify the Lpd-GFP–expressing cells visualized in the pseudocolor panels beneath. Relative intensity of the Lpd-GFP fusion are indicated using the pseudocolor scale shown at top. (B) Quantitative analysis used the average GFP intensity along a tail-to-head vector [illustrated in far right panels of (A)] to calculate the intensity ratio in the front 20% of the cell relative to the rear 80%. Representative GFP intensity plots are shown, and graph on the right represents the average front/rear ratios of 10 Lpd WT-GFP and 10 ΔPH cells. (C) Side view of migrating cells, showing the distribution of Lpd-GFP or Lpd ΔPH-GFP versus PI(3,4)P2. The Lpd WT-GFP or Lpd ΔPH-GFP RAJI cells migrating under a SDF-1 gradient were fixed at the end of 100 min of time-lapse imaging, stained for PI(3,4)P2, and analyzed by digital reconstruction of Z-stack confocal images. Original magnification ×63. Images shown are renderings of the X–Z plane (perpendicular to the glass substrate) at the cellular median through both cell body and uropod. Two representative cells for each Lpd-GFP and Lpd ΔPH-GFP are shown. Similar localization patterns were observed in 10 cells randomly examined for each cell type.
including early genetic studies (61) and recent studies applying Btk inhibitor as a therapeutic for B cell malignancies (62). The overlapping biological and therapeutic effects of PI3Kδ or Btk inhibitors in CLL led to acceptance of a simplified model for PI3K signaling in CLL that only considers PIP3 and Btk. Our results suggest that PI(3,4)P2 and its binding proteins also need to be considered as functionally important components of the PI3K pathway in malignant B cells, and likely normal B cells as well. It is possible that the clinical benefit of PI3Kδ inhibitors in CLL may be mediated in part by reducing PI(3,4)P2 and impairing function of PI(3,4)P2-binding proteins such as Lpd. Because PI(3,4)P2 may also be generated by G protein–coupled receptor–coupled class I PI3K β and γ isoforms and class II PI3Ks (23, 26, 63, 64), and is controlled by distinct PI phosphatases, more effective inhibition of malignant B cell migration might be achieved by considering therapeutic strategies targeting PI(3,4)P2 signaling in addition to PIP3 signaling.

**FIGURE 7.** Lpd mediates chemotaxis of malignant B cells in a PH domain–dependent manner. (A) RAJI cells were transfected or lentivirally transduced with empty control (Ctrl) vector or vector encoding Lpd WT-GFP for overexpression (Lpd-GFP) or encoding shRNA targeting the coding sequence (KD-CDS) or 3′ untranslated region (KD-UTR) of endogenous Lpd. Left panel, Lpd protein KD was confirmed by three Western blots for each of the shRNAs, and representative blots are shown. Right panel, Cells were assayed for migration toward SDF-1 in Transwell chambers. Data represent mean ± SD of at least four independent experiments per condition and are expressed as normalized migration relative to the control cells (with empty or nonsilencing shRNA vectors). (B) Cells transfected with empty vector (Ctrl), Lpd KD-UTR vector (KD), KD plus Lpd WT-GFP, or KD plus Lpd ΔPH-GFP were assayed for chemotaxis in microfluidic devices. Cell migration tracks are shown. (C and D) Migration speed and chemotactic index were calculated from cell tracks. Bars represent the mean ± SEM based on 77, 98, 81, and 77 cells for the four above-mentioned groups, respectively, pooled from two independent experiments. **p < 0.01, ***p < 0.001, ****p < 0.0001.
Disclosures

The authors have no financial conflicts interest.

References

Supplementary Figure 1. Flow cytometry controls for anti-PI(3,4)P2 staining
The indicated cells were stimulated with SDF-1 for 5 min or left unstimulated, fixed, permeabilized and stained with the indicated primary Abs. After incubation and washing, all samples were stained with Alexa647-labelled secondary antibodies and analyzed by flow cytometry. The Alexa647 mean fluorescence intensity (MFI) is shown and represents the average and SEM of replicates.
Supplementary Figure 2. Expression of INPP4A and INPP4B in human B cell lines and primary CLL. Protein extracts from the indicated cell lines and CLL cells isolated from eight different patients were blotted to detect INPP4A or INPP4B. Positive control lanes (4A or 4B control) contain extracts from RAJI cells expressing INPP4A or 4B respectively. Note that a fivefold shorter exposure is shown for the 4B control lane, in order to allow clear visualization of the over-expressed protein band. Membranes were re-probed for GAPDH as a loading control.
Supplementary Figure 3. PH domain-dependent colocalization of Lpd and PI(3,4)P2 is apparent throughout the depth of the migrating cell.
RAJI cells expressing Lpd WT-GFP or Lpd ΔPH-GFP were subjected to an SDF-1 gradient, fixed and stained to detect PI(3,4)P2. Confocal images were acquired at different distances from the coverslip (Z positions). Colocalization of PI(3,4)P2 and Lpd WT-GFP or Lpd ΔPH-GFP was quantified using Pearson’s correlation coefficient. For each transfectant type results from one representative cell at multiple Z positions are shown.
Video 1. Migration of RAJI cells expressing inactive INPP4A. Cells were loaded onto a microfluidic chemotaxis device that maintains a 100 nM SDF-1 gradient with higher SDF-1 concentration at the top. Time lapse images were converted to video using Image J.

Video 2. Migration of RAJI cells expressing active INPP4A. Video was generated under the same conditions as Video 1.